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Attenuation of Human Immunodeficiency Virus Type 1 Cytopathic Effects by Replacing a 424-bp Region of Envelope from a Noncytopathic Biological Clone

SERENE E. FORTE, MOHAN SOMASUNDARAN, and JOHN L. SULLIVAN

ABSTRACT

We analyzed the *env* genes of cytopathic and noncytopathic biological clones derived from two HIV-1-infected children with discordant clinical courses. Chimeric viruses were constructed by switching *env* regions from V2 through V3 of the biological clones with the corresponding region from the molecular clone NL4-3. These HIV-1 chimeric viruses exhibited similar replication kinetics as well as syncytium-inducing abilities. The chimeric virus containing the *env* region of noncytopathic biological clone, GC6 8-4, was noncytopathic in an *in vitro* cell-killing assay, while the chimeric virus containing the *env* region of cytopathic biological clone, HC4, was cytopathic in the *in vitro* cell-killing assay. These studies suggest the presence of a cytopathicity determinant that maps to the envelope sequences contained within the downstream region of V2 and within the V3 region (nucleotide position 6822 to nucleotide position 7250, based on NL4-3 sequence).

INTRODUCTION

THE ENTRY of human immunodeficiency virus type 1 (HIV-1) into cells is mediated by the envelope glycoprotein. HIV-1 strains differ in their ability to infect specific cell types and can be characterized *in vitro* according to their ability to replicate in macrophages and established T-cell lines as well as by their ability to induce cytopathic effects. Two groups of isolates have been described: those that are capable of replicating in established T-cell lines (T-cell tropic) and those that infect primary macrophages (M-tropic). T-cell tropic isolates replicate more rapidly and to higher levels in primary lymphocytes (rapid/high) than M-tropic isolates, which replicate slowly and to lower levels in primary lymphocytes (slow/low).^{1–4} Many isolates that infect T cells are able to induce syncytia and are termed syncytium-inducing (SI), whereas most M-tropic isolates are non-syncytium-inducing (NSI).

Recently, new discoveries have shown that chemokine receptors are important cofactors for entry of HIV-1 into cells.⁵ CXCR4 mediates viral entry into CD4-positive T-cell lines and CCR5 mediates entry into macrophages.^{6–10} Dual tropic HIV-1 strains have been identified that can use either one of these receptors to gain entry into cells.^{6,8}

The hallmark of HIV-1 infection is the progressive destruction of CD4 T cells *in vivo*.^{11,12} Macrophage tropic NSI iso-

lates are preferentially transmitted and are present throughout the length of infection.^{13–15} The emergence of T-cell tropic SI isolates correlates with the decrease of CD4 lymphocytes and progression of HIV-1 infection to AIDS.^{4,16} However, this switch to T-cell tropic SI isolates is not necessary for T-cell depletion and progression to AIDS, especially in vertical HIV-1 infection.^{17,18}

There is no clear correlation between when an infant is infected and disease progression. It has been reported that an association exists between *in utero* infection, high viral load in the early neonatal period, and rapid disease progression.¹⁹ However, plasma RNA levels in infants infected *in utero* or in the intrapartum period are not easily separable. While it is true that most infants with high viral burdens will exhibit disease progression, we have followed vertically infected children for several years with extremely high viral loads and normal CD4 counts.^{17,18} While disease progression following vertical infection may be variable with observable differences in the occurrence of illness and survival times,²⁰ by and large vertically infected children experience a more rapid clinical course than adults. A number of factors are likely to be responsible for the observed variability in clinical course. These include the route of perinatal HIV-1 transmission,²¹ the viral burden,^{22,23} the biological phenotype,^{15,24–26} and host factors including both cellular and humoral immune responses.^{27–29}

The molecular mechanism(s) of HIV-1-induced cell lysis remains unclear and is likely to be multifactorial. Differences in HIV-1 viral tropism, phenotype, and replication kinetics have been assigned to the envelope surface glycoprotein.^{30,31} Along with these properties, studies of mutant HIV-1 viruses have provided evidence that a major determinant for single cell killing maps to the viral envelope glycoproteins.^{32–34} Cao et al.³⁵ described an inducible system to examine envelope-mediated single cell lysis. Their studies demonstrated that the ability of HIV-1 envelope glycoproteins to mediate membrane fusion was obligatory for both syncytium formation and single cell lysis.

Previously, we described functional HIV-1 biological clones that were isolated from two infected children with high viral loads *in vivo*¹⁸ who had discordant clinical courses. The absolute CD4 cell counts of HIV-1-seropositive pediatric patients A and D, at the time of virus isolation, were 2312 and 64 counts/mm³, respectively. The primary HIV-1 isolate, A, obtained from patient A at 47 months of age, was NSI and HIV-1 isolate, D, obtained from patient D at 36 months of age, was SI. Patient D has subsequently succumbed to AIDS, while patient A remained asymptomatic (and is currently on combination antiretroviral therapy). Multiple biological clones were obtained from HIV-1 isolates A and D. All the biological clones from isolate A were NSI and all the biological clones from isolate D were SI in MT-2 cells. Representative biological clones, GC6 8-4 (derived from isolate A obtained from patient A) and HC4 (derived from isolate D obtained from patient D) grew to the highest titer and were selected for further characterization.¹⁸ These clones exhibited similar replication kinetics but differed in syncytium-inducing ability: biological clones GC6 8-4 and HC4 are NSI and SI, respectively. Furthermore, the biological clones GC6 8-4 and HC4 differed markedly in *in vitro* cell-killing assays: GC6 8-4 was noncytopathic and HC4 was cytopathic.¹⁸

To assess the relationship between envelope gene sequences and cell killing, we analyzed the envelope gene of biological clones GC6 8-4 and HC4. Chimeric viruses were constructed by switching the envelope sequences contained within the downstream region of V2 and within the V3 region of each of the biological clones with the corresponding region from the molecular clone NL4-3. These chimeric viruses exhibited similar replication kinetics as well as syncytium-inducing abilities. The only difference was in their ability to induce cell killing. These studies provide evidence that in certain viral variants, HIV-1-induced cytopathicity maps to the envelope sequences contained within the downstream region of V2 and within the V3 region (*StuI* at position 6822 to *NheI* at position 7250 based on NL4-3 nucleic acid sequence³⁶).

MATERIALS AND METHODS

Patients

Patient A was clinically asymptomatic when the virus was isolated and remains so 7 years later. At the time of viral isolation, patient A was 47 months old, with normal CD4 counts of 2312/mm³. Unexpectedly, peripheral viremia in this child was very high as determined by serum HIV-1 p24 antigen levels (1773 pg/ml) and by plasma viral RNA copies (280,000/ml).

Since the original viral isolate was obtained, Patient A has maintained normal CD4 T-cell numbers and is currently on combination antiretroviral therapy. Patient D had AIDS at the time of viral isolation. The child was 36 months old with very low CD4 T cell counts (64/mm³).¹⁸

Virus culture

Peripheral blood mononuclear cells (PBMC) from seronegative individuals were infected with biological clones, GC6 8-4 and HC4.¹⁸ On days 3 and 5 of viral infection, culture supernatants were monitored for HIV-1 p24 antigen production by ELISA. On day 5 postinfection, 10⁷ cells were harvested and genomic DNA was extracted.

DNA isolation

PBMC (10⁷) from cultures infected with biological clones, GC6 8-4 and HC4, were pelleted and washed twice with phosphate-buffered saline (PBS). Genomic DNA was prepared by resuspending the cell pellets in 5 ml lysis buffer (10 mM Tris-HCl, 400 mM NaCl, 2 mM EDTA, pH 8.2) to which sodium dodecyl sulfate (SDS) (final concentration 0.7%) and proteinase K (1.7 mg) were added. The genomic DNA preparation was incubated at 37°C overnight. Following this incubation, 1.6 ml saturated NaCl was added, mixed, and centrifuged at 2500 rpm for 15 min. To the supernatant, 7 ml of isopropanol was added to precipitate the DNA. The DNA was pelleted by centrifugation, resuspended in 30 μ l TE, and stored at -20°C.

PCR amplification of *env* region

Approximately 200 ng of genomic DNA was used as a template for amplification of a 3.0-kb product of HIV-1 proviral sequence containing the entire coding region for gp160. The reaction mixture contained 10 μ l GeneAmp PCR Buffer II (50 mM KCl, 10 mM Tris-HCl); 2.5 mM MgCl₂; 200 μ M each of dATP, dCTP, dGTP, and dTTP; 10 pmol each of primer I5-1 (5'-CAGAATTGGGTGTCGACATAGCAG-3') and MN12 (5'-CACTTGCCACCCATCTTATAGGAAAATCCTTTC-CAAGCC-3') in a final volume of 90 μ l. Primers I5-1 and MN-12 anneal at positions 5774–5797 and 8762–8801, respectively, based on published NL4-3 nucleotide positions.³⁶ Polymerase chain reactions (PCR) were completed in an automated Perkin-Elmer (GeneAmp 9600) thermal cycler programmed for the following: denaturation at 94°C for 3 min, followed by the addition of Perkin Elmer AmpliTaq (2.5 U in 10 μ l) during 87°C hold ("hot start"). The reactions were subjected to 30 cycles at 94°C for 1 min, 55°C for 1.5 min, and 72°C for 3 min. A final extension step at 72°C for 10 min was added after the last amplification cycle.

Molecular cloning of *env* region into an expression vector

To screen for the appropriate sized amplicon, 10 μ l of the final PCR amplification was resolved by electrophoresis on a 0.8% agarose gel. The remaining 90 μ l of the reaction was phenol-chloroform extracted, ethanol precipitated, resuspended in 15 μ l of water, and resolved by electrophoresis on a 0.8% agarose gel. The 3-kb DNA fragment was excised from the gel,

purified by Elu-Quick (Schleicher & Schuell, Keene, NH), resuspended in water, and stored at -20°C until further use. The purified product was ligated into pBluescript (Stratagene, La Jolla, CA) and 10 μl of the ligation reaction was used to transform DH5 α bacterial cells. Transformed colonies were color selected on ampicillin, IPTG (isopropyl- β -D-thiogalactopyranoside), X-gal (5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside) plates. White colonies were chosen and amplified in culture. Minipreps were performed from the overnight cultures. The miniprep DNA was screened for the appropriate sized insert by restriction digest and electrophoresis on a 0.8% agarose gel.

DNA sequencing of full length env region

The double-stranded plasmid DNA was sequenced in both the forward and reverse directions with primers that spanned the entire env regions overlapping every 200 bps (primer sequences available upon request). Of double-stranded DNA template 1 $\mu\text{g}/\mu\text{l}$ was added to 8 μl terminator premix (A-dye terminator, C-dye terminator, G-dye terminator, T-dye terminator, dTTP, dATP, dCTP, dTTP, Tris-HCl, pH 9.0, MgCl₂, thermal stable pyrophosphatase, and AmpliTaq DNA polymerase FS), 10 pmol of primer, and water to a final volume of 20 μl . DNA sequencing was performed in an automated Perkin-Elmer thermal cycler (GeneAmp 9600) programmed for 30 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1.5 min, and extension at 72°C for 3 min. The reaction mixture was ethanol precipitated to remove unincorporated deoxynucleoside triphosphate and oligonucleotides, and then vacuum dried. The dye-deoxy-labeled sequencing reaction mixes were resolved by electrophoresis on a 6% polyacrylamide gel and analyzed with an automated DNA sequencer (model 373A, Applied Biosystem, Inc., Foster City, CA). Individual sequences were assembled by using the Sequencher program (Gene Codes Corp, Ann Arbor, MI). Sequences were determined for both strands of DNA and were aligned by the methods of Lamers et al.³⁷ at Gene Genie, Inc (Thibodaux, LA).

Plasmids

Plasmid pNL4-3, containing a chimeric HIV-1 provirus, was obtained from the AIDS Research and Reference Reagent Program, Rockland, MD. The subclones, p83-2 and p83-10, the 5' and 3' half of NL4-3, respectively, were kindly provided by R. Desrosiers.³⁸

PCR of the *StuI* to *NheI* region of env

Since GC6 8-4 and HC4 envelope sequence lack the *NheI* sites but contain the *StuI* sites, this region was obtained by means of PCR with *StuI* primer (5'-ATTACACAGGCCTGTCCACAGGTA-3') and *NheI* primer (3'-TTCTCTTAATTTGCTAGCTATCTG-5'). Approximately 200 ng of DNA was used as a template for amplification of a 424-bp product of HIV-1 env sequence contained within the downstream region of V2 and within the V3 region. The reaction mixture contained 10 μl GeneAmp PCR Buffer II (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 2.5 mM MgCl₂, 200 μM each of dATP, dCTP, dGTP, and dTTP, and 10 pmol each of primer, in a final volume of 90 μl . "Hot start" PCR reactions were completed in an automated Perkin-

Elmer (GeneAmp 9600) thermal cycler programmed for denaturation at 94°C for 3 min, followed by the addition of AmpliTaq (2.5 U in 10 μl) during 87°C hold. The reaction mixtures were subjected to 30 cycles at 94°C for 1 min, 55°C for 1.5 min, and 72°C for 3 min. A final extension step at 72°C for 10 min was added after the last amplification cycle.

Molecular cloning of NL4-3 chimeric constructs, p83-SFG and p83-SFH

From the PCR reaction 10 μl was resolved by electrophoresis on a 1% agarose gel to screen for the appropriate molecular weight amplicon. The remaining 90 μl of the PCR reaction mixture was ethanol precipitated and resuspended in 15 μl of water and stored at -20°C . The 424-bp DNA fragment was gel purified by Elu-Quick, phenol-chloroform extracted, ethanol precipitated, and resuspended in water. The purified product was cloned into p83-10³⁸ by appropriate restriction sites (*StuI* at position 6822 and *NheI* at position 7250 based on published NL4-3 nucleotide sequence).³⁸ Aliquots (10 μl) of the ligation product was used to transform competent DH5 α bacterial cells. Colonies were chosen at random and amplified overnight in 5 ml LB broth containing ampicillin. A small scale plasmid preparation was performed to recover DNA. Of the plasmid preparation 10 μl was digested with appropriate restriction endonuclease, and the presence of an insert was screened by electrophoretic resolution on a 1% agarose gel.

Cell culture

CEM \times 174 cells and Jurkat cells were maintained in medium containing RPMI-1640 (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (GIBCO Laboratories, Grand Island, NY). Jurkat-CCR5 cells (kindly provided by M. Emerman) were maintained in RPMI-1640/10% FCS containing 0.2 $\mu\text{g}/\text{ml}$ of puromycin. PBMC and CD4 T cells were cultured at 2×10^6 cells/ml in RPMI-1640/10% FCS containing 10 units/ml of recombinant human interleukin-2 (Boehringer-Mannheim, Indianapolis, IN).

Transfections

Transfections were carried out following the procedure described by Gibbs et al.³⁸ Briefly, just prior to transfection, 3 μg of DNA was added stepwise in five aliquots with gentle vortexing to 1.4 ml of Dulbecco modified Eagle medium (DMEM) DEAE-Dextran solution (DMEM containing 125 $\mu\text{g}/\text{ml}$ of DEAE-Dextran in 50 mM Tris, pH 7.3). In a 15-ml conical tube, 6 ml of CEM \times 174 was pelleted containing $0.5\text{--}0.8 \times 10^6$ cells/ml, which were split the night prior. The cells were washed twice with 5 ml of serum-free DMEM and resuspended in 1.4 ml of DNA-DMEM-DEAE-Dextran solution. The cell DNA-DMEM mixture was incubated at 37°C for 1 h. After incubation, the cells were pelleted and washed with 5 ml serum-free DMEM. Cells were resuspended in 6 ml RPMI-1640/10% FCS and incubated at 37°C . Supernatant p24 antigen activity was monitored every 2-3 days. Cells were split as needed 1:2 or 1:3 twice weekly.

Viral phenotype of HIV-1 chimeric viruses

Chimeric viruses (SF-G 1, 3, 4, 5 and SF-H 2, 3, 4, 5) were phenotyped on MT-2 cells for their ability to replicate and form

A) V1-V2

conb	NCITDL	MAT	NTNSS	NTNMEKGEIKNCSFNITTSIRDQKVQKEYALFYKLDVVPID	NST	YRLISC
HC4	---	TS	S---	SE-AKGG--M--N--N--K-----Q--	D-	TS-----
cdc42	---NTN	Y	TT	ELSIIVVWEQRG--MR--R-----E--DNK	T	NNTK---N-
sc	---N-R	D	ST-AT	NTTSSNRGK--G--MT-----S-----	TS	-T--N-
brva	---H-F			SNSGK-ME-GE-----M-----I--NDKT	TR	
mn	---R	T	---N-TANWNS	NSEGTIGK--M-----M-----L--I-S-	DS	TS-----
nl43	K---K	D		SGRMI-----S-----F--I--	TS	---
sf33	---YLG		---N	SGGTV--E-----G-----A--Y--DDNT	TS	---H-
sf2	---G	K		NWKEEI-----I--N--RN-	AS	TTTNYI N---H-
han	K---YN			WGR---Q---KV--N--S---T---N	K-SNRDNTI	S-M--H-
rf	---ANL	G	VT	SSSGG-M--N---QV--R--T--K-----EKG	IS	PKNNTSNNTSYGN-T--H-
gun	---N-V		YS	WEK-DR---Q---RK-N--Q--LNR--I--	KTS	-T--T-
lai	K---G		NTNSS	SGEMM-----S--G-----F--II--	D-	TS-T-T-

GC68-4	YNY	CS	ATEA	CTKANNIS	KEMI	E--	M	HRP-I	NS	SNTS	
jrcsf	--K-V		TT	SSSEGM--R--K-----NK	N				N		K-----
jrfl	--K-V		TT	NDS EGT--R-----E-----NN	TS				TS		
bal1	---R		G-DT	NTTSSSRGMVGG--M--N--G-----IA-	NS				NS		NNR-----
yu2	---R		TT	SSSWET-----N-----	AS				AS		
ada	---R	V	I-N	SEGMR-----K-D--R--ND	TS				TS		---N-
sf162	--IN-K			NTKSSNWKE-DR---KV--N-M-----ND	TS				TS		-K--N-
jfl	--IN-K	V	I-N	WGK--E---T--KN-M-R-----KD	TS				TS		-T--N-

B) V3

conb	FTDNAKTIIVQLNESVEINCTRPNNTKRSIH	IGPGRAFYTTGEIIGDIRQAHNCISRAKWNNTLKQIVTKLREQFG	NKTIVFNQSSGGD
HC4	---M-----A-----R-RIGH---V---G-----T--D--R-V-R---H-		---I---
cdc42	---N-----V-----H--RVT---VW---L-N-----Q---Q-A-T---		---A---
sc	---K-A-----TR-----A-D-----I--D-E---		---I--R---
brva	---N-V-----R-T---M---VY---Q---R---L-S-E---V-K---		---R---
mn	---H-----Q---Y-K--R---KN--T---D--R--S-K--K---		---K---
nl43	---T-----RIQ R---V-I-K--NM---A--AS---N---I-K---		---N---
sf33	---N-----L-V---R-RR-T---S--KVL---K-Y---K-E-VA---		---K---
sf2	---N-----A---Y---H--R---K---E-K---N---		---N---
han	---T-----H---G---V---R-V---L---R-K-N-FR---IRQFE---		---R---
rf	---V-----A-Q---T---K---VI-A-Q---K---L-Q---V---D---		---TS---
gun	---N-----IV---T---S---HAIEK--N---KEN---		---K---
lai	---Q-----RIQ R---V-I-K--NM---A--AS---N---I-K---		---N---

GC68-4	V	S	R	V-AVT	KTE-Y	VAE-G	
jrcsf	---K	S			Q	E	N
jrfl	---K				D	I	E
bal1	---V				L	D	NK-I
yu2	---N	V	N		L	KTQ-E	E-AI-K
ada	---N	K			T	N-A	K-N
sf162	---K		T		A-D	GE	QA
jfl	---M	K	IA		L	VQ	I

C) V4-V5

conb	CNSTQLFNSTWN	NGT	NNT	TNDT	NTITLPCRICKIINHWQEVGKAMYAPPIRGQIRCSSNITGLLLTRDGG	NGT	EIFRP
HC4	-DT-A-		DIERA	EG-D	L-----V-----G-----V	K	NEA-T-T-
cdc42	---A-	V	S-G	WSVTRKQKDT##DI	-----R--V-----L-K-L-----GE	G	T-----
sc	---S-		EGS	GGND	-----E-----K-VK-----NSK	H	KNENT---
brva	---YR	T	G-I	EGNSP	-----K-----MN	E	TDT---
mn	---TSP	G	WN-T	GSNN-I	-Q-K-----E-----KDDT	D	---
nl43	---F	S	WSTEGS	EGSD	-Q-K-F-----S-----NNN	S	---
sf33	---T-K	E	S	G	-----E-----GDK	S	T-----
sf2	---T	N-RL	H	EGTKG-D	-I-----G-S-----T	V	NDT-V---
han	---K		N	STWNDNG-D	-I-----G-L-----I	ND	SS TT---
rf	---T		S	EGS	-----V-----S-K-I-----EDTT	T	---
gun	---T	LV	W	KRS	---Q-----S-----Q-----	S	ENT---
lai	---F	S	WSTEGS	EGSD	-----F-----S-----NNN	S	---

GC68-4	D	R	DGTNT	N	SDT-D	Q	N-S	N	N	NGT
jrcsf	---		D	EKSSGTEG-D		I	K	E	S	EI
jrfl	---		N	EGS	VE-N	EG		INE		
bal1	---		V	EES	GR-I		R	PED	K	V
yu2	---		D	RKL	QS-G-EGNDT			KDT		
ada	---		F	W-L	-G-		I	T	SS	GS
sf162	---		N	IGP			R	KEIS	T	
jfl	---	LS		W-D	EESKDTREP-I		R	N	KE	A

syncytia following a previously published protocol.³⁹ The protocol was modified to determine viral replication in MT-2 cells using antigen capture ELISA to measure HIV-1 p24 gag protein in cell-free supernatants.¹⁷ Similarly, chimeric viruses were phenotyped on Jurkat cells and Jurkat-CCR5 cells for their ability to form syncytia and to replicate.

Infection of Jurkat-CCR5 cells with chimeric viruses, SF-G1 and SF-H2

Since both SF-G1 and SF-H2 chimeric viruses infected and replicated in Jurkat-CCR5 T cells and not in Jurkat T cells, Jurkat-CCR5 cells were infected with HIV-1 chimeric viruses according to previously described methods.¹⁸ HIV-1 infected and uninfected Jurkat-CCR5 cells were cultured at 0.5×10^6 cells/ml in RPMI-1640/10% FCS containing 0.2 μ g/ml of puromycin.

Infection of primary PBMC with HIV-1 chimeric viruses

Of viral p24 from chimeric viruses obtained by transfections 50 ng/ml was used to infect PBMC. Briefly, normal PBMC from HIV-1-seronegative donors were phytohemagglutinin (PHA) stimulated for 3 days and infected with HIV-1 chimeric viruses, SF-G1 and SF-H2, according to previously described methods.¹⁸ HIV-1-infected and uninfected PBMC were cultured at 2×10^6 cells/ml in RPMI-1640/10% FCS containing 10 units/ml of recombinant human interleukin-2.

Depletion of CD8 T cells

PBMC were isolated by Ficoll density and CD8 T cells were depleted using anti-CD8 antibody-coated magnetic beads (Dyna, Inc., Lake Success, NY) according to manufacturer's protocol. The supernatant containing CD4 T cells was centrifuged and the cell pellet was resuspended in RPMI-1640/10% FCS with 10 units/ml of interleukin-2.

Infection of CD4 T cells with HIV-1 chimeric viruses, SF-G1 and SF-H2

CD4 T cells were PHA stimulated overnight and infected with HIV-1 chimeric viruses, SF-G1 and SF-H2, according to previously described methods.¹⁸ HIV-1-infected and uninfected CD4 T cells were cultured at 2×10^6 cells/ml in RPMI-1640/10% FCS with 10 units/ml of interleukin-2.

Assessment of HIV-1-induced cytopathic effects

HIV-1-induced cytopathic effects by the chimeric viruses, SF-G1 and SF-H2, were determined by evaluating cell viability using trypan blue dye exclusion.

Measurement of cell surface CD4 and CD8 expression

Cell surface CD4 and CD8 expression was measured by flow cytometry. Briefly, 10^7 cells from either infected or uninfected T-cell cultures were washed with cold PBS containing 1% azide and incubated for 30 min on ice with a mixture of fluorescein-labeled anti-CD4 monoclonal antibody and phycoerythrin-labeled anti-CD8 monoclonal antibody (Becton-Dickinson, San Jose, CA). The cells were washed with PBS containing 1% azide and fixed for 10 min in 0.2% paraformaldehyde in PBS and analyzed by FACscan flow cytometer (Becton-Dickinson, San Jose, CA). Phycoerythrin- and fluorescein-labeled mouse gamma-globulins were used as negative stains for controls.

Determination of HIV-1 proviral copies

To determine the number of proviral copies of HIV-1 viral DNA, PCR was performed on lysates of serially diluted CD4 T cells infected with chimeric viruses, SF-G1 and SF-H2, using the Roche Amplicor HIV-1 test kit following the previously published method.⁴⁰ Briefly, a region of HIV-1 *gag* gene was amplified using biotinylated SK431/SK462 primer pairs in an automated Perkin Elmer (GeneAmp 9600) thermocycler. The cycling parameters were as follows: five cycles of denaturation at 95°C for 10 sec, annealing at 55°C for 10 sec, and extension at 72°C for 10 sec; followed by 30 cycles of denaturation at 90°C for 10 sec, annealing at 60°C for 10 sec, and extension at 72°C for 10 sec, and an additional extension at 72°C for 5 min. The amplicons were captured in wells coated with the probe and detected by ELISA using avidin-conjugated horseradish peroxidase as a secondary marker.

Measurement of viral replication

To measure HIV-1 replication in infected PBMC, Jurkat-CCR5 cells, and CD4 T-cell cultures, cell-free culture supernatants were assayed for HIV-1 p24 gag protein using an antigen capture ELISA.

RESULTS

Derivation of HIV-1 biological clones and chimeric viruses

The derivation of HIV-1 biological clones and NL4-3 chimeric viruses from viral isolates obtained from patients A and D are delineated below:

Patient A → isolate **A** → clone **GC6 8-4** → chimeric GC68-4/NL4-3 (**SF-G1**)

Patient D → isolate **D** → clone **HC4** → chimeric HC4/NL4-3 (**SF-H2**)

FIG. 1. Alignment of variable regions of the envelope gene of HIV-1 biological clones, GC6 8-4 and HC4, and T-cell and macrophage tropic viruses. (A) Alignment of *env* V1/V2 T-cell tropic (top) and macrophage tropic (bottom) viruses. (B) Alignment of V3 T-cell tropic (top) and macrophage tropic (bottom) viruses. (C) Alignment of *env* V4/V5 T-cell tropic (top) and macrophage tropic (bottom) viruses. The highlighted regions show the potential N-linked glycosylation sites within the variable region of the envelope protein. GC6 8-4 has lost two potential N-linked glycosylation sites within V1/V2, one in V3 and within V4/V5 regions. The sequences derived from HC4 and GC6 8-4 clearly align with the published SI and NSI viral sequences, respectively. This is in agreement with our phenotypic assignment of HC4 as an SI virus, and GC6 8-4 as an NSI virus.

Env sequences from HIV-1 biological clones

Figure 1 shows the variable regions (V1-V5) from HIV-1 biological clones, HC4 and GC6 8-4, aligned to SI/T-cell tropic and NSI/macrophage tropic viruses, respectively. HIV-1 biological clone, GC6 8-4, has lost two potential N-glycosylation sites within V1-V2, one in V3 and two within V4-V5 regions.

The V3 region derived from HC4 clearly aligns with published SI viral sequences. This is in agreement with our phenotypic assignment of HC4 as an SI virus. Similarly, the V3 region of GC6 8-4 aligns with published NSI viral sequences and agrees with our assessment of GC6 8-4 as an NSI virus.¹⁸

V3 sequences determined from chimeric viruses

Zhong et al.⁴¹ and others⁴² have shown a correlation between overall amino acid change and the phenotype of HIV-1 viruses. V3 loops of SI and NSI variants generally have an overall charge of +5 and +3, respectively. Moreover, T-cell tropic SI viruses generally have a basic amino acid at one or more of the following positions: 11, 24, 25, and 32 (numbering from the

first cysteine of V3). Macrophage tropic NSI viruses have either an acidic amino acid or alanine at position 25. The third variable region of envelope sequenced from HIV-1 chimeric viruses SF-G1 and SF-H2 are 35 and 38 amino acids in length, respectively (Fig. 1B). The V3 region from HIV-1 chimeric virus SF-H2 carries an overall charge of +7 with a basic amino acid located at position 11 of the V3 loop (Fig. 1B). Zhong and colleagues⁴¹ report that a switch to arginine at position 11 along with a switch to glycine at position 25 coincides with a change from NSI to SI phenotype. The V3 region from HIV-1 chimeric virus SF-H2 has these changes (Fig. 1B). The V3 region from HIV-1 chimeric virus SF-G1 carries an overall charge of +5 with an alanine at position 25 of the V3 loop (Fig. 1B).

Phenotype of chimeric viruses

Chimeric viruses were made by removing the region from *StuI* (nucleotide 6822) to *NheI* (nucleotide 7250) from pNL4-3 and replacing it with the corresponding region from each biological clone (Fig. 2A). To assess if the switch in regions from

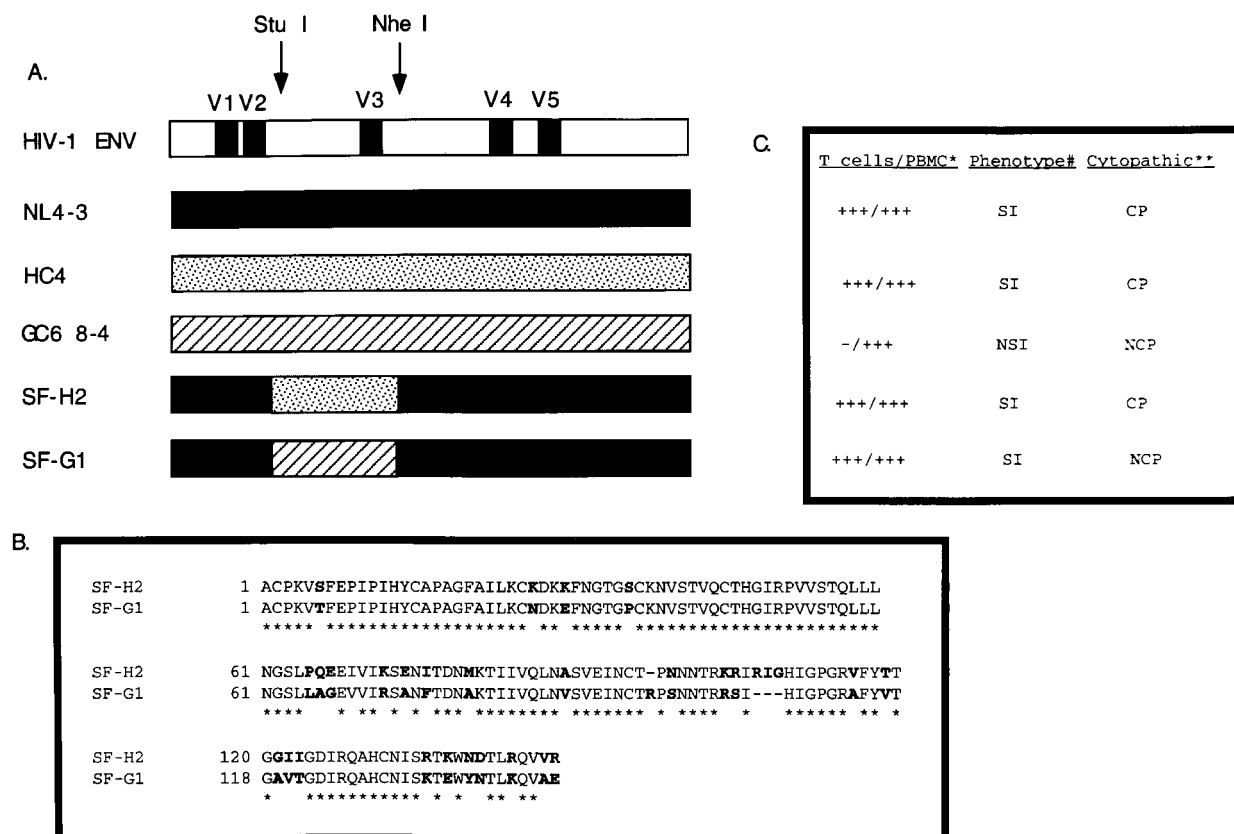


FIG. 2. Construction of chimeric viruses, SF-G1 and SF-H2. (A) A schematic representation of the chimeric cloning strategy. Chimeric viruses, SF-G1 and SF-H2, were constructed using the molecular clone, NL4-3, as the backbone. The 424-bp insert that was cloned into NL4-3 was obtained by PCR from the *env* gene isolated from the biological clones, HC4 and GC6 8-4, and cloned into pBluescript expression vector (p83-14 and p83-23, respectively). (B) The amino acid sequence from *StuI* to *NheI* (nucleotide position 6822 to 7250, based on NL4-3 sequence) from *env* gene isolated from the biological clones, HC4 and GC6 8-4, and cloned into pBluescript expression vector. The highlighted region shows the amino acids that are different between the two chimeric viruses, SF-G1 and SF-H2. (C) The tropism, phenotype, and cytopathic profiles of the constructs. *+, +, viral replication in T-cell lines and/or PBMC; -, no viral replication; # SI, syncytium-inducing; NSI, nonsyncytium inducing; **CP, cytopathic in T-cell lines and PBMC; NCP, noncytopathic in T-cell lines and PBMC.

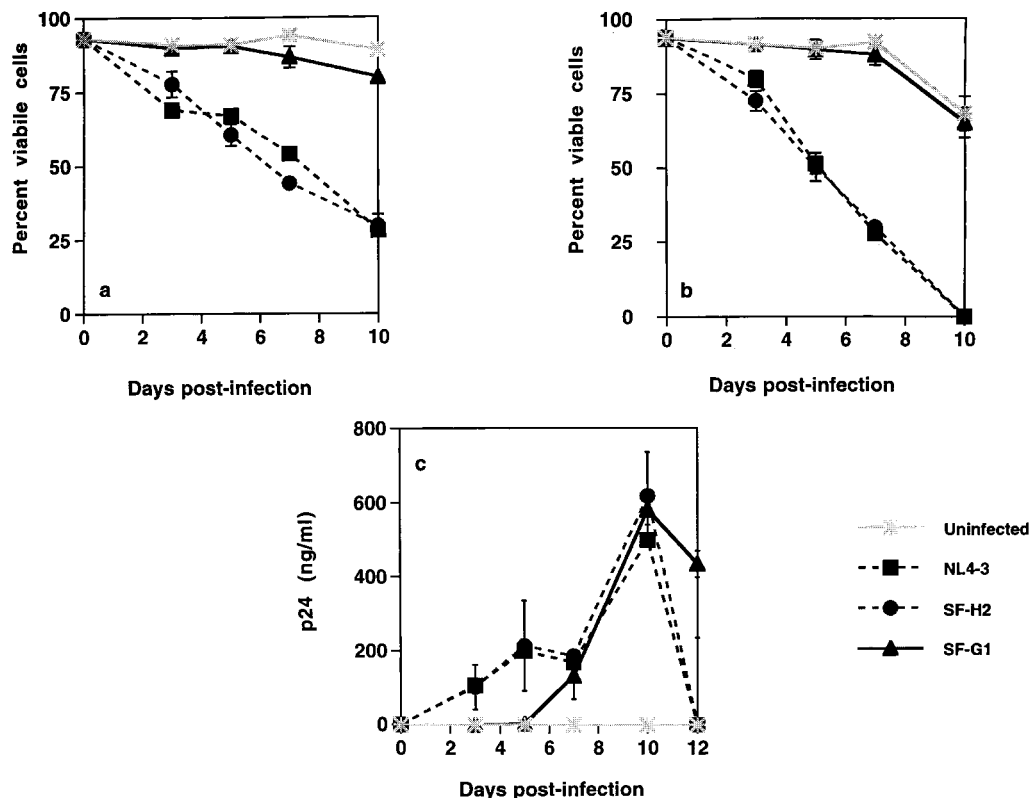


FIG. 3. Viability of PBMC and CD4-enriched T cells infected with HIV-1 chimeric viruses, SF-G1 and SF-H2. Mitogen-stimulated PBMC or CD4-enriched T cells were infected with titrated chimeric viruses, SF-G1 and SF-H2, as described in Materials and Methods. Cells underwent complete media change every 2 days. Percent viable cells was calculated every 2 days by trypan blue dye exclusion. HIV-1 replication in infected PBMC and CD4 T-cell cultures was measured every 2 days by assaying cell-free culture supernatants for HIV-1 p24 gag protein using an antigen capture ELISA. Data from eight individual experiments are presented along with standard deviations. (a) Viability of PBMC infected with chimeric viruses, SF-G1 and SF-H2. (b) Viability of CD4-enriched T cells infected with chimeric viruses, SF-G1 and SF-H2. (c) Replication kinetics of chimeric viruses, SF-G1 and SF-H2, in infected CD4-enriched T cells.

StuI to *NheI* had any effect on the phenotype of the chimeric viruses, the ability of each chimeric virus to form syncytia was measured in a standard MT-2 assay. The removal of the region from pNL4-3 between *StuI* to *NheI* and replacement with the *StuI* to *NheI* region (Fig. 2B) from HIV-1 biological clones, HC4 and GC6 8-4, did not change the SI viral phenotype of the parental virus, NL4-3 (Fig. 2C). Both SF-G1 and SF-H2 viruses formed syncytia in MT-2 cells. This is in contrast to the previous result with the biological clone, GC6 8-4, which is NSI in MT-2 cells.¹⁸

In similar assays, the chimeric viruses SF-G1 and SF-H2, and the NL4-3 virus replicated and induced syncytia in Jurkat-CCR5 cells. However, NL4-3 virus and SF-H2 chimeric virus and not SF-G1 chimeric virus replicated to high titers in Jurkat cells.

Infection of primary PBMC with chimeric HIV-1, SF-G1 and SF-H2

PHA-stimulated seronegative donor PBMC were infected with HIV-1 chimeric viruses SF-H2 or SF-G1. The infection of PBMC with the two HIV-1 chimeric viruses showed notably different outcomes. The pattern of cell lysis in PBMC infected

with HIV-1 chimeric virus SF-H2 virus was very similar to the control cytopathic infection with NL4-3 virus. The results were observed for six separate experiments. On day 5 postinfection, the mean percent viability of PBMC infected with HIV-1 chimeric virus SF-H2 was 60 (± 4) and PBMC infected with NL4-3 was 62 (± 16) (Fig. 3a). By day 10 postinfection, 70% of the PBMC were dead in the cultures infected with either the HIV-1 chimeric virus, SF-H2, or the known cytopathic molecular clone, NL4-3 (Fig. 3a). In contrast, PBMC infected with SF-G1 retained a level of viability comparable to the uninfected control cells. On day 5 postinfection, the mean percent viability of PBMC infected with SF-G1 was 90 (± 2) and the uninfected control was 91 (± 1) (Fig. 3a). By day 10 postinfection, the mean percent viability of uninfected PBMC or PBMC infected with SF-G1 was 90 (± 1) and 80 (± 2), respectively (Fig. 3a).

Replication kinetics of chimeric HIV-1, SF-G1 and SF-H2, in PBMC

The replication kinetics of both viruses were similar to each other as well as to NL4-3. There was a delay in viral p24 production by HIV-1 chimeric virus SF-G1 but this was transient

and eventually the viral replication was equivalent to that of SF-H2 and NL4-3. These results were observed for six separate experiments. On day 5 postinfection, the mean p24 assayed from the cultures infected with HIV-1 chimeric viruses, SF-H2 and SF-G1, was 120 (± 119) and 49 (± 94) ng/ml, respectively. By day 7 postinfection, the mean p24 assayed from the cultures infected with HIV-1 chimeric viruses, SF-G1 and SF-H2, was 148 (± 54) and 206 (± 81) ng/ml, respectively.

Infection of CD4-enriched T cells with chimeric HIV-1, SF-G1 and SF-H2

CD4-enriched T cells were infected with HIV-1 chimeric virus SF-H2 or HIV-1 chimeric virus SF-G1. The infection of CD4-enriched T cells with the two chimeric viruses showed notably different outcomes. The pattern of cell destruction in CD4-enriched T cells infected with HIV-1 chimeric virus SF-H2 was very similar to the control cytopathic NL4-3 virus. On day 5 postinfection, the mean percent viability (obtained from eight separate experiments) of CD4-enriched T cells infected with SF-H2 was 53 (± 2) and CD4 enriched T cells infected with NL4-3 was 52 (± 5) (Fig. 3b). By day 7 postinfection, approximately 70% of the CD4-enriched T cells were dead in the cultures infected with either HIV-1 chimeric virus SF-H2 or NL4-3 (Fig. 3b). However, HIV-1 chimeric virus SF-G1 was phenotypically different from SF-H2. On day 5 postinfection, the mean percent viability of CD4-enriched T cells infected with HIV-1 chimeric virus SF-G1 was 89 (± 4) and the uninfected control was 91 (± 2) (Fig. 3b). By day 7 postinfection, approximately 90% of the CD4-enriched T cells were viable from cultures either uninfected or infected with HIV-1 chimeric virus SF-G1 (Fig. 3b).

Replication kinetics of chimeric HIV-1, SF-G1 and SF-H2, in CD4-enriched T cells

The replication kinetics of both viruses were similar to the NL4-3 virus. There was a delay in viral p24 production by HIV-1 chimeric virus SF-G1 but this was transient and by day 7, SF-G1 viral replication (132 \pm 63 ng/ml) was equivalent to that of HIV-1 chimeric virus SF-H2 (186 \pm 18 ng/ml) and NL4-3 (Fig. 3c). By day 10 postinfection, the mean p24 assayed from the cultures infected with HIV-1 chimeric viruses, SF-H2 and SF-G1, was 617 (± 158) and 580 (± 40) ng/ml, respectively (Fig. 3c). These results were observed for eight separate experiments. Viral infection was also assessed by measuring the amount of proviral DNA. Quantitative DNA PCR analysis demonstrated that the proviral copy numbers were similar in CD4-enriched T cells infected with either HIV-1 chimeric virus SF-H2 or HIV-1 chimeric virus SF-G1 (data not shown).

Outcome of CD4 T cells infected with chimeric HIV-1, SF-G1 and SF-H2

To determine the fate of CD4-enriched T cells infected with HIV-1 chimeric viruses, SF-G1 and SF-H2, flow cytometric analyses were performed on cells removed from the infected cultures at days 3, 5, 7, and 10 postinfection. The percentage of CD4 T cells in uninfected and HIV-1 biological clone infected cultures was determined by flow cytometry (Fig. 4). The percentage of CD4 T cells in HIV-1 chimeric SF-H2-infected

cultures dramatically declined over 5 to 7 days postinfection. Between days 3 and 5 postinfection, CD4 T cells declined from 82 to 21% (Fig. 4d). By day 7 postinfection, the percentage of CD4 T cells decreased to 15 and by day 10 the culture was completely dead. In contrast, by day 5 postinfection, there was 82% CD4 T cells in HIV-1 chimeric virus SF-G1 infected cultures (Fig. 4b), and 90% CD4 T cells in the uninfected culture (Fig. 4a). The cells in the lower left quadrant represent viable T cells that have down-regulated their CD4 antigen expression due to viral infection. On day 7 postinfection, 72% of T cells in HIV-1 chimeric virus SF-G1 infected culture expressed CD4 antigen and 20% of viable T cells had down-regulated their expression of CD4 antigen.

Infection of Jurkat-CCR5 cells with the chimeric viruses, SF-G1 and SF-H2

To address the question of whether the chimeric virus, SF-G1 was truly attenuated or whether the HIV-1-induced cell lysis was delayed, we used a T-cell line, Jurkat-CCR5. One limitation with the use of CD8-depleted PBMC cultures is that the life of these cells *in vitro* is very short (10–12 days) compared with T-cell lines that are adapted for culture and can be kept in culture indefinitely. Jurkat-CCR5 T cells were infected with HIV-1 chimeric virus SF-H2 or HIV-1 chimeric virus SF-G1. The infection of Jurkat-CCR5 T cells with the two chimeric viruses showed notably different outcomes. The pattern of cell destruction in Jurkat-CCR5 T cells infected with HIV-1 chimeric virus SF-H2 was very similar to the control cytopathic NL4-3 virus. On day 8 postinfection, the percent viability of Jurkat-CCR5 T cells infected with SF-H2 was 56% and Jurkat-CCR5 T cells infected with NL4-3 was 46% (Fig. 5a). The viral replication on day 8 postinfection, as measured by p24 antigen production, was equivalent for all the three viruses, NL4-3, SF-H2, and SF-G1 (186, 230, and 317 pg/ml, respectively (Fig. 5b). Viral replication peaked on day 10 with cultures infected with NL4-3, (1178 ng/ml), SF-H2 (1486 ng/ml), and SF-G1 (1141 ng/ml) producing equivalent amounts of p24 antigen (Fig. 5b). By day 13 postinfection, approximately 80% of the Jurkat-CCR5 T cells were dead in the cultures infected with either HIV-1 chimeric SF-H2 or NL4-3 (Fig. 5a). The cultures infected with either SF-H2 or NL4-3 were completely dead by day 17 postinfection. In contrast, infection of Jurkat-CCR5 T cells with HIV-1 chimeric virus SF-G1 produced different results. The differences between the uninfected control and those T cells infected with SF-G1 were observed early in the course of the infection. Between days 8 and 15 postinfection, the viability of T cells infected with SF-G1 ranged from 65% (day 8) to 60% (day 15). During this time many syncytia were observed in the cultures infected with NL4-3, SF-H2, and SF-G1. Interestingly, on day 17 postinfection the cultures infected with SF-G1 began to recover and 74% of the T cells were viable (Fig. 5a). On day 22 postinfection, the percent viability of Jurkat-CCR5 T cells infected with HIV-1 chimeric virus SF-G1 was 90% as was the uninfected control (Fig. 5a). From day 20 through the end of the experiment on day 38, the culture infected with SF-G1 continued to produce virus (figure 5b). On day 39 postinfection when the experiment was terminated, 97% of the Jurkat-CCR5 T cells were viable from cultures either uninfected or infected with HIV-1 chimeric virus SF-G1 (Fig. 5a).

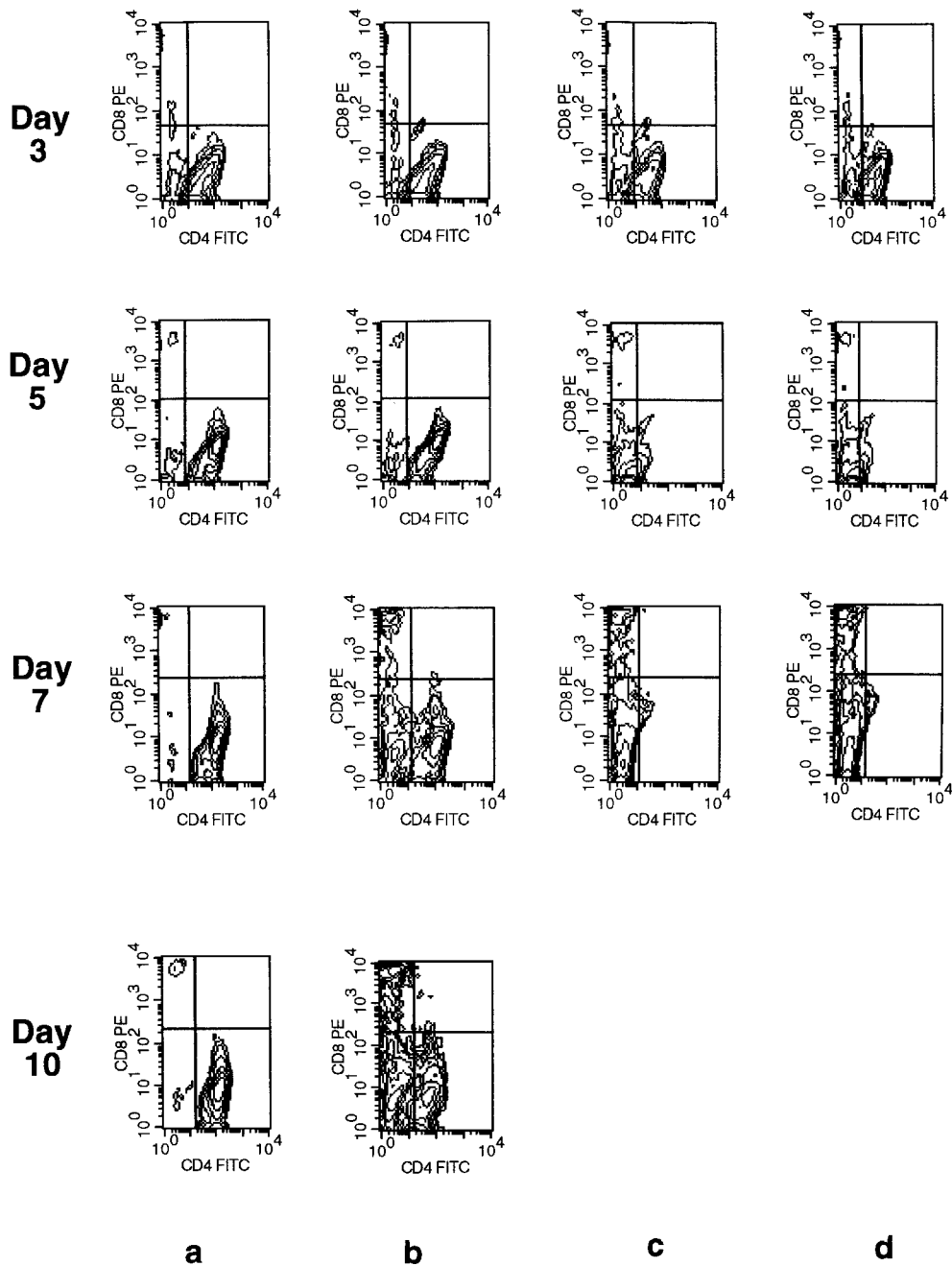


FIG. 4. CD4-enriched T cells infected with the chimeric virus, SF-G1, retain viable CD4-positive cells. Two-color flow cytometry analysis of CD4-enriched T cells uninfected or infected with either NL4-3 virus or chimeric viruses, SF-G1 and SF-H2. Cells were harvested on days 3, 5, 7, and 10 postinfection, stained for both CD4 and CD8, fixed in paraformaldehyde, and analyzed by flow cytometry. (a) Uninfected. (b) Chimeric virus, SF-G1. (c) Chimeric virus, SF-H2. (d) Molecular virus clone, NL4-3.

On day 24 of the infection, cell-free viral supernatant from the SF-G1 culture, now termed SF-G1R1, was used to infect new Jurkat-CCR5 T cells. Between days 3 (day 27) and 6 (day 33) postinfection, the viability of T cells reinfected with SF-G1R1 ranged from 75% (day 29) to 60% (day 33) (Fig. 5a). During this period many syncytia were observed in the cultures. Interestingly, on day 7 postinfection with SF-G1R1 the infected cultures began to recover and 75% of the T cells were viable (SF-

G1R1, Fig. 5a). On day 13 postinfection, the percent viability of Jurkat-CCR5 T cells reinfected with HIV-1 chimeric virus SF-G1 was 89% (SF-G1R1, Fig. 5a).

DISCUSSION

In this study we genetically analyzed a specific region within HIV-1 envelope (nucleotide positions 6822 to 7250) from a cy-

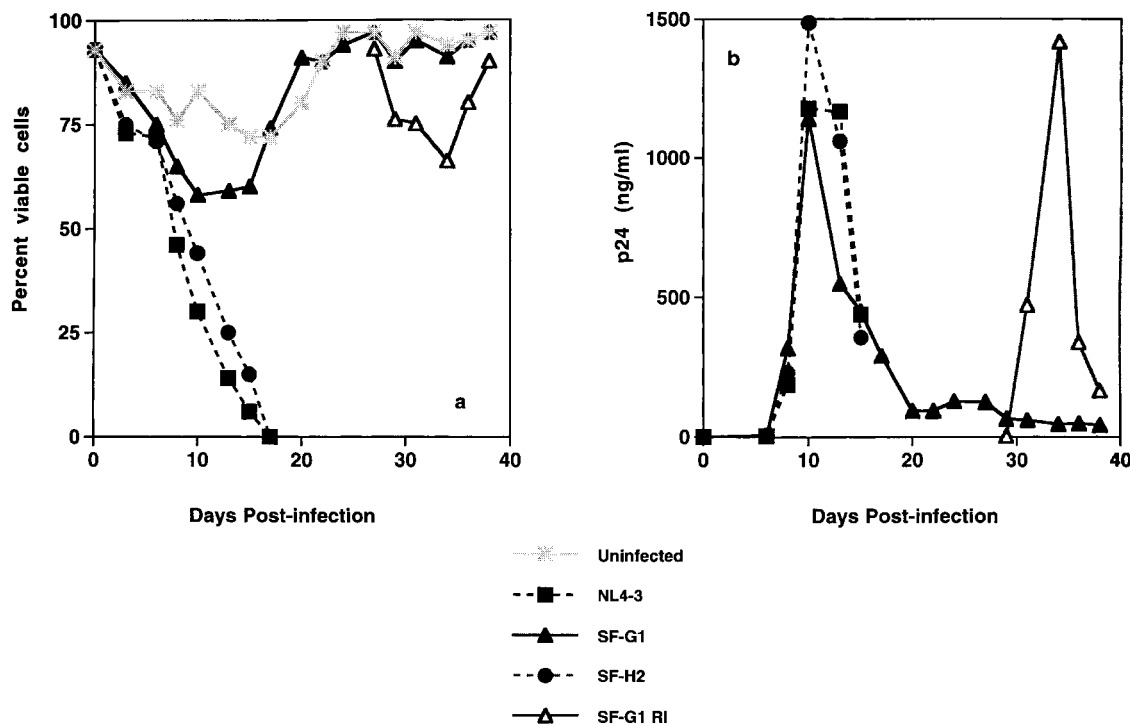


FIG. 5. Viability of Jurkat-CCR5 cells infected with HIV-1 chimeric viruses, SF-G1 and SF-H2. Jurkat-CCR5 cells were infected with titrated chimeric viruses, SF-G1 and SF-H2, as described in Materials and Methods. Cells underwent complete media change every 2 days. On day 24 of the infection, cell-free viral supernatant from the SF-G1 culture, now termed SF-G1R1, was used to infect fresh Jurkat-CCR5 cells. Percent viable cells was calculated every 2 days by trypan blue dye exclusion. HIV-1 replication in infected Jurkat-CCR5 cultures was measured every 2 days by assaying cell-free culture supernatants for HIV-1 p24 gag protein using an antigen capture ELISA. Data from one representative experiment are presented. (a) Viability of Jurkat-CCR5 cells infected with chimeric viruses, SF-G1 and SF-H2. On day 24 of the infection, cell-free viral supernatant from the SF-G1 culture, now termed SF-G1R1, was used to infect fresh Jurkat-CCR5 cells. (b) Replication kinetics of chimeric viruses, SF-G1 and SF-H2, in Jurkat-CCR5 T cells.

topathic and a noncytopathic biological clone by generating chimeric viruses with the well-characterized molecular clone, NL4-3. The most striking difference between these chimeric viruses when compared to each other as well as NL4-3 was that SF-G1, the chimeric virus containing a downstream V2 and V3 region of the noncytopathic envelope, was attenuated for cytopathicity. This switch in cytopathicity held true in PBMC as well as in T-cell lines (Figs. 3 and 5).

In general, viruses that infect macrophages are restricted in their ability to infect T-cell lines. It has been shown that viruses isolated early in infection are NSI and macrophage tropic, while viruses recovered from individuals with low CD4 T-cell counts are generally SI with the ability to replicate in T-cell lines.¹⁻⁴ When the phenotype of each chimeric virus was assessed it was found to be identical to the parental virus NL4-3, which is SI. The chimeric viruses, SF-G1 and SF-H2, were both SI despite different V3 signature sequences that predicted that SF-G1 would be NSI and SF-H2 would be SI. The V3 region sequenced from SF-H2 has classic SI hallmarks, such as increased charge (+7, Fig. 1B) and a switch from an arginine at position 11 along with a switch to glycine at position 25 (Fig. 1B). The predicted phenotype based solely on the V3 sequence of SF-H2 is SI. The results are in agreement with this prediction (Fig. 2C). At all stages in the viral population, i.e., quasispecies,¹⁷ biological

clone,¹⁸ and chimeric virus (this report), the predicted phenotype was the determined phenotype. In contrast, the V3 region sequenced from SF-G1 showed features attributed to viruses having an NSI phenotype, such as an overall change of +5 with an alanine at position 25 of the V3 loop (Fig. 1B). Therefore, the predicted phenotype, based solely on the V3 sequence of SF-G1, was NSI. SF-G1 chimeric virus replicated to high titers in Jurkat-CCR5 T cells and not in Jurkat T cells. The phenotype of the patient A quasispecies was NSI¹⁷ as was the phenotype of the biological clone isolated from the quasispecies.¹⁸ Although the chimeric clone SF-G1 preferentially used the CCR5 coreceptor the phenotype of the SF-G1 virus was SI (Fig. 2C). These results contrast with previous published observations^{30,31} that show that mutations within the envelope gene (gp120), allowing an SI phenotype, are capable of increasing cytopathicity. However, these data are consistent with the recent published observation in the SIV system.¹ Kimata and Overbaugh showed that the minimal region of SIV responsible for syncytium formation mapped to the envelope gene.⁵² Even though the phenotype of the chimeric SIV env was SI it did not increase the cytopathicity in either CEM×174 or macaque PBMC. Other variable regions besides V3 have been suggested to play a role in HIV cytopathicity. It has been shown that envelope glycoproteins that contain either site-directed mutations

within the V1/V2 region^{43,44} or V1/V2 regions containing chimeric V2 sequences exhibit altered cytopathicity.^{45–47} Sullivan and co-workers demonstrated that specific amino acids mutations in the tip of V2 yielded fusion defective viruses.⁴⁴ In addition, all of the mutant V1/V2 envelope glycoproteins displayed decreased syncytium-inducing abilities, with some mutants lacking the ability to induce cell fusion altogether. Taken together, these results demonstrate that the third variable region of envelope is not the only region involved in syncytium formation. Moreover, the formation of syncytium does not necessarily dictate cell death (Fig. 5). In other words, the NSI/SI phenotype does not correlate with the NCP/CP phenotype. These results further support previous observations that HIV-1-induced single cell killing is independent of syncytium formation.⁴⁸

Differences in HIV-1 viral tropism, phenotype, and replication kinetics have been assigned to the envelope surface glycoprotein.^{30,31} Along with these properties, studies of mutant HIV-1 viruses have provided evidence that a major determinant for cell killing maps to the viral envelope glycoproteins. For these reasons, prior to analyzing these subgenomic fragments, we attempted to construct full-length env chimeric viruses by replacing the env region from NL4-3 with either the HC4 or GC6 8-4 env gene. The many attempts using standard cloning techniques, *in vitro* recombination, and advanced cloning techniques failed to develop a stable full length functional envelope product. For reasons we can not explain, envelope genes isolated from these biological clones are extremely unstable.

The molecular mechanism(s) of HIV-1-induced cell lysis is likely to be multifactorial. Experiments with mutant HIV-1 viruses do not unequivocally resolve the role of viral accessory proteins (Vif, Vpr, Tat, Nef) in HIV-1 cell killing.^{49–51} Cheng-Mayer and colleagues concluded from their observations with recombinant viruses that the envelope region contains the major determinants of HIV-1 cytopathicity.³² Stevenson et al.³⁴ reported that two amino acid changes within gp120 altered the efficiency of processing of the gp160 envelope glycoprotein precursor, which in turn decreased the ability of a replication-competent HIV-1 virus to induce single cell lysis. Kowalski et al.³³ showed that a mutation within the gp41 amino terminus of envelope, which generated a replication-competent noncytopathic virus, was attenuated in its ability to form syncytia and to induce single cell death. Cao et al.³⁵ described an inducible system to examine envelope-induced single cell lysis. Their studies demonstrated that the ability of HIV-1 envelope glycoproteins to mediate membrane fusion was obligatory for both syncytium formation and single cell lysis.

Our results demonstrate that the cytopathic profile of NL4-3 can be altered by a specific disruption of the envelope sequences contained within the downstream region of V2 and within the V3 region (nucleotide positions 6822 to 7250). Interestingly, disrupting this region by inserting the same region from a cytopathic biologically cloned virus (HC4) did not alter the ability of the parental virus to induce cell death (Fig. 2). It was only when that region was disrupted with the insertion of the same region from a noncytopathic biologically cloned virus (SF-G1) did the cytopathic profile change (Fig. 2). These results suggest that it is not just the disruption of that region but the disruption of that region by a specific sequence, which attenuates the cytopathic profile of the parental virus. The pre-

cise role of these specific sequences in cytopathicity and coreceptor usage will be further analyzed using detailed mutagenesis studies that are underway.

Cao et al.³⁵ and others^{31–34} have shown that differences in HIV-1 cytopathicity can be mapped to mutations within the envelope glycoproteins. Recent observations regarding SIV-induced cytopathicity have reached similar conclusions. Kimata and Overbaugh⁵² showed that SIV-induced cytopathicity is determined by mutations in *gag* and *env* genes. Karlsson et al.⁵³ using passaged recombinant SHIV-89.6P viruses constructed between SIV (SIV_{mac}239) and HIV-1 (89.6) showed that changes within the 3' half of the SHIV-89.6P genome that occurred within the envelope gene were responsible for increased cytopathicity. Taken together, these findings, along with those reported herein, support the hypothesis that the HIV-1 envelope glycoprotein plays a role in HIV-1-induced cytopathicity. Further studies addressing the role of envelope in HIV-1-induced cell killing are necessary to understand the specific molecular mechanism(s) involved in CD4 T-cell depletion in AIDS pathogenesis.

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